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Biocontrol and Plant Pathogenic *Fusarium oxysporum*-Induced Changes in Phenolic Compounds in Tomato Leaves and Roots

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Abstract

The biocontrol fungus *Fusarium oxysporum* strain CS-20 was previously shown to reduce the incidence of Fusarium wilt of tomato through an uncharacterized host-mediated response. As phenolic compounds are involved in the defence response of tomato to pathogens and other stressors, this work was undertaken to determine whether biocontrol strains induced changes in phenolic compounds in leaves and roots of tomato seedlings in the presence and absence of pathogenic *F. oxysporum* f. sp. *lycopersici*. Roots of intact tomato seedlings were placed in water or aqueous fungal spore suspensions. Two biocontrol *F. oxysporum* strains [CS-20 (host-mediated mechanism) and 85SK-1 (control mechanism unknown)] and two plant pathogenic strains of *F. oxysporum* f. sp. *lycopersici* Race 1 were used. After 24 or 72 h exposure, phenolic compounds were extracted from leaves and roots before identification by HPLC. There were significant qualitative and quantitative differences between the two sampling times. Compared with the control treatment, strain CS-20 significantly altered (usually increasing) the ferulic, caffeic and vanillic acid contents, and concentrations once unidentified phenolic compounds recovered from leaves and roots. In another experiment, tomato seedlings growing in sterile sand were drenched with spores of strain CS-20 the day before treating them with varying concentrations of spores of the pathogen for 24 or 72 h. The amount of pathogen present did not significantly affect the plant phenolic response to the presence of strain CS-20. This work demonstrates that tomato responds within 24 h to the presence of the biocontrol strain CS-20 by alterations in secondary metabolism that are typical of resistance responses in tomato.

Introduction

The fungus *Fusarium oxysporum* Schlecht. is found in soils worldwide. While most strains are saprophytic, others cause a vascular wilt disease on crops ranging from vegetables to banana and date palms, and still other strains can protect plants from wilt. The pathogenic strains are highly host specific and are identified to *formae speciales* and race. The disease is managed through the fumigation of soil with broad-spectrum biocides, particularly methyl bromide, and through host resistance. The removal of methyl bromide from the market due to environmental concerns about currently-available fumigants, and the occasional emergence of new races of the pathogen have created interest in the development of alternative disease management strategies. Among the strategies being investigated are biocontrol and induced resistance.

Fusarium wilt of tomato is caused by *F. oxysporum* f. sp. *lycopersici*. The pathogen occurs as three races. Host resistance to the pathogen consists of physical barriers in the roots, as well as active defence responses within the plant (May, 1930; Beckman, 1987). Phenolic compounds cause the vascular browning symptom characteristically associated with Fusarium wilt (Mace et al., 1972) and have also long been associated with resistance of tomato to Fusarium wilt (Menon and Schachinger, 1957), as well as many other host–pathogen interactions (Nicholson and Hammerschmidt, 1992; Hammerschmidt, 2005). Phenolic compounds are well known for their roles in plant defence responses, both as preformed inhibitors and those formed in response to attack by pathogens (Nicholson and Hammerschmidt, 1992). Phenolic compounds are both antimicrobial (Gómez-Vásquez et al., 2004; Jung et al., 2004), and critical to host defence in sensing and defence-triggering (Beckman, 2000). The rapid release of compartmentalized phenolic compounds may be responsible for the host reaction leading to the formation of tyloses and gums that restrict movement of wilt pathogens within the vascular system (Beckman, 1987, 2000).

There are numerous reports of non-pathogenic *F. oxysporum* or pathogenic strains on non-hosts controlling other Fusarium diseases (Blok et al., 1997; Elmer, 2004; Forsyth et al., 2006; Nel et al., 2006; Olivain et al., 2006, and reviewed in Mandeel and Baker, 1991; Martyn and Biles, 1991; Fravel et al., 2003; He and Wolyn, 2005). Our previous work identified *F. oxysporum* strain CS-20 for the management of Fusarium wilt of tomato, watermelon, muskmelon and basil (Larkin et al., 1996; Larkin and Fravel, 1999a, 2002a, 2002b). A glucose competition assay showed that competition was a less important mechanism for strain CS-20 than for strain Fo47 (Larkin and Fravel, 1999b). Split-root tests demonstrated that strain CS-20 reduces the incidence of Fusarium wilt of tomato through a host-mediated mechanism (Larkin and Fravel, 1999b). Other examples of non-pathogenic *F. oxysporum* reducing disease via a host-mediated mechanism have been reported (Duijff et al., 1998; Huertas-González et al., 1999). Understanding the nature of this resistance could aid in the development of alternative disease management tools for Fusarium wilt. This work was initiated to compare the accumulation of phenolic compounds in tomato after exposure to biocontrol and plant pathogenic strains of *F. oxysporum*.

Materials and Methods

In vitro production of phenolic compounds by *Fusarium oxysporum* strains

Four *Fusarium* strains were used in experiments. Two biocontrol *F. oxysporum* strains [CS-20 (host-mediated mechanism) and 85SK-1 (control mechanism unknown)] and two plant pathogenic strains of *F. oxysporum* f. sp. *lycopersici* Race 1 (32SK-3 and 34SK-3) (Bao et al., 2004) were used. Fungi were grown in 1% soy hull fibre (Hebbar et al., 1996) for 4–5 weeks with shaking at ~80 r.p.m. under ambient conditions. The contents of each replicate flask were divided into three parts. One part contained both mycelia and culture filtrates mixed in a Waring blender. The remaining two-thirds was filtered through coarse filter paper to separate mycelia from culture filtrates for the other two parts. All three samples types had 6 ml of anisic aid solution (1 μg/ml; Aldrich W39430, St. Louis, MO) and 4 ml of 0.1% acidified water (125 μl H_3PO_4/l water) added. The homogenate was then centrifuged twice at 4000 × ***g*** for 10 min.

Strata-X reverse phase columns (Phenomenex 8B-S100-FBJ; Phenomenex, Torrence, CA) were prewashed with 3 ml acidified sterile distilled water (SDW), 3 ml of methanol (Sigma 646377, HPLC grade, St Louis, MO), and another 3 ml of acidified SDW. The supernatant of the homogenate was then vacuum filtered through the column at approximately −80 kPa. The column was washed with 10 ml of acidified SDW, and then the sample was eluted with 3 ml of methanol. Forty microlitres of 10% phosphoric acid was added to the sample. The 3 ml sample was evaporated to ~150 μl with a Büshi RE 111 Rotavapor (Newcastle, DE) with a Büshi 461 water bath operating at 50°C. Samples were assayed immediately.

Phenolic compounds were separated by C_{18} RP-HPLC using a Waters model 2690 HPLC system (Milford, MA) with data acquisition by a Dell Pentium 4 computer (Baker et al., 2005). A 200 × 4.6 mm i.d., 5 μm Onyx Monolithic C18 analytical column (Phenomenex) was used with a binary mobile phase of methanol in 0.01% aqueous phosphoric acid as previously described (Baker et al., 2005). Sample aliquots of 100 μl were acidified with 0.1% phosphoric acid and placed in an autosampler with a 30-μl injection volume. The gradient began with 5% methanol in 95% acidified water (v:v), increased to 30% methanol after 20 min and 60% methanol at 40 min after injection before rinsing the column for the next sample. Quantification of peak height was performed using the UV_{max} wavelength for each peak and reported as relative HPLC units per gram of plant tissue in the original sample. The experiment was repeated.

Effect of pathogenic and biocontrol *F. oxysporum* on production of phenolics in tomato – biocontrol root dip

Seeds of tomato [*Solanum lycopersicum* L. cv. Bonny Best (susceptible to Race 1); seed not treated with fungicide] were surface disinfested in 5% NaOH for 3 min before rinsing in SDW. Seeds were placed on a moist filter paper in 150 mm × 15 mm petri plates. When plants were approximately 5 cm in total length (8–11 days), seeds were transferred to 98-cell plastic seedling trays (3 cm × 3 cm × 7 cm tall cells) filled with sterile sand (Sakrete fine natural sand) After planting, each seedling was drenched with 5 ml Murashige–Skoog nutrient solution (Gibco, Grand Island, NY) and the seedling trays placed under intermittent mist to maintain moisture.

When there were four true leaves (approx. 4–6 weeks), 10–15 plants per treatment were gently removed from the sand and the roots were washed in SDW three times to remove the sand. Roots of intact plants were placed in 250 ml beakers with either 50 ml water or an aqueous suspension of spores and mycelia of one of four fungal strains (CS-20, 85SK-1, 32SK-3 or 34SK-3) to cover the plant roots but not the leaves. Fungal suspensions were prepared from fungi grown in 1% soy hull fibre as described above. Mycelia and growth media were homogenized with sterile Waring blenders. The resulting suspension was a mixture of all spore types (primarily microconidia) plus mycelia. After counting spores on a haemacytometer, spore concentration was adjusted to 10^5 spores (all spore types)/ml SDW. After 24 or 72 h in water or spore suspension, up to 3 g of tomato leaves and roots was homogenized separately in 12 ml of anisic acid solution (1 μg/ml) plus 8 ml of 0.1% acidified water (125 μl H_3PO_4/l water). Plant tissue was weighed before homogenation (0.5–3 g). The phenolic compounds were extracted from the homogenate as described above. The resulting sample was evaporated with a Rotavapor, as above. Known samples of phenolic compounds were also tested under these conditions and retention times were compared to those from plant tissue for tentative

identification of some compounds recovered from plant tissue. The experiment was conducted eight times and each repeat of the experiment was considered a replicate. Data were analysed by analysis of variance in SAS (SAS, Cary, NC, USA).

Dosage response – biocontrol drench

The response of plants to the biocontrol strain CS-20 was studied in the presence of varying concentrations of the pathogen. Plants and fungi were grown as above. The day before plant harvest, 4- to 6-week-old seedlings were drenched with 5 ml of either SDW or a 10^5 spores/ml solution of biocontrol *F. oxysporum* strain CS-20. This drenching method was used for previously reported field and greenhouse tests with strain CS-20 (Larkin and Fravel, 1999a,b; Larkin et al., 1999; Larkin and Fravel, 2002a,b). After 24 h, seedlings were gently removed from the sand and the roots were washed in SDW three times. Plants were placed in beakers of water or fungal suspensions as described above. The pathogen used was *F. oxysporum* f. sp. *lycopersici* Race 1 (32SK-3). The fungal suspension was a mixture of mycelia and 10, 10^3 or 10^5 spores/ml, consisting of all spore types (primarily microconidia).

After 24 or 72 h, plant roots and leaves were collected and the phenolic components extracted as above with the following exceptions to enhance the recovery of phenolics. This experiment was carried out at 4°C, and the samples were kept on ice at all times. The tissue was harvested and homogenized as above except 30 ml of 0.2% phosphoric acid was used, resulting in a sample pH of 2–2.5. The anisic acid standard was not added at this time for this experiment. After homogenation, sample preparation was the same as above except a Waters Sep Pak Vacuum Manifold at −80 kPa was used to process the samples. The column was washed with 5 ml 0.2% acidified water (250 μl H_3PO_4/l water). Before evaporation, 200 μl 0.1 M anisic acid was added as an internal standard. Finally, the 3 ml sample was reduced until almost dry under nitrogen gas. Each sample was then brought back up to 150 μl with a 1 : 1 mixture of methanol and water. The experiment was conducted three times and each repeat of the experiment was considered a replicate. The height of the HPLC peak per gram of tissue in the original sample was analysed by linear regression in SAS.

Results

Phenolic compounds from fungi

In an experiment to determine whether phenolic compounds were produced directly by the fungi *in vitro*, trace amounts of vanillic acid were recovered from all four fungi. Trace amounts of other phenolic compounds were occasionally recovered from fungi.

Effect of pathogenic and biocontrol *F. oxysporum* on production of phenolics in tomato – root dip

Relative to the control treatment, phenolic compounds produced in both tomato roots and leaves were quantitatively affected at 24 and 72 h of exposure to two pathogenic and two biocontrol strains of *F. oxysporum* strains (Tables 1 and 2). The greatest changes observed were in ferulic, caffeic and vanillic acids, as well as in an unidentified phenolic compound with an HPLC profile of 324–327.6 nm (UN-324). The amount of ferulic acid recovered from the leaves was significantly reduced by 24 h exposure to the biocontrol strain CS-20 and one of the plant pathogenic strains (Table 1). There were no differences among treatments for ferulic acid recovered from leaves for 72 h exposures. The amount of caffeic acid recovered from leaves after 24 h exposure to each of the four fungi was significantly greater than from plants treated with water alone (Table 1). After 72 h exposure, significantly more caffeic acid was recovered from the leaves of plants treated with one pathogenic strain than from the water treatment and less was recovered from leaves treated with the biocontrol strain 85SK-1.

The amount of ferulic acid recovered from roots was significantly greater from plants treated with either of the pathogenic strains of *F. oxysporum* compared with the water treatment (Table 2). With 72 h exposure to the fungi, significantly more ferulic acid was

Table 1
Phenolic compounds from tomato leaves[a]

Time exposed to fungus	Ferulic acid			Caffeic acid			Vanillic acid		
	Treatment	Peak height	Duncan group	Treatment	Peak height	Duncan group	Treatment	Peak height	Duncan group
24 h	Water	0.381	A	85SK-1	0.651	A	Water	0.277	A
	85SK-1	0.295	AB	32SK-3	0.508	B	85SK-1	0.215	AB
	32SK-3	0.259	AB	CS-20	0.504	B	CS-20	0.213	AB
	34SK-3	0.212	B	34SK-3	0.483	B	34SK-3	0.199	B
	CS-20	0.211	B	Water	0.349	C	32SK-3	0.182	B
72 h	32SK-3	0.244	A	34SK-3	0.493	A	43SK-3	0.312	A
	CS-20	0.242	A	32SK-3	0.441	AB	Water	0.305	A
	85SK-1	0.224	A	Water	0.420	AB	85SK-1	0.279	AB
	34SK-3	0.210	A	CS-20	0.328	BC	32SK-3	0.273	B
	Water	0.157	A	85SK-1	0.287	C	CS-20	0.170	B

[a]Bare root tomato seedlings were placed in beakers with spore suspensions of pathogenic (32SK-3 or 34SK-3) or biocontrol (CS-20 or 85SK-1) strains of *F. oxysporum*. After 24 or 72 h, phenolic compounds were extracted from roots and leaves. These were identified and quantified (relative units, peak height) by HPLC.

Table 2
Phenolic compounds recovered from tomato roots[a]

Time exposed to fungus	Ferulic acid			Caffeic acid			Vanillic acid		
	Treatment	Peak height	Duncan group	Treatment	Peak height	Duncan group	Treatment	Peak height	Duncan group
24 h	34SK-3	0.412	A	85SK-1	0.186	A	32SK-3	0.424	A
	32SK-3	0.388	AB	Water	0.152	AB	85SK-1	0.423	A
	CS-20	0.288	BC	32SK-3	0.136	AB	34SK-3	0.401	A
	Water	0.268	C	34SK-3	0.115	AB	Water	0.362	A
	85SK-1	0.257	C	CS-20	0.087	B	CS-20	0.328	A
72 h	CS-20	0.487	A	32SK-3	1.636	A	34SK-3	0.437	A
	34SK-3	0.454	A	Water	1.301	A	85SK-1	0.406	AB
	85SK-1	0.396	A	34SK-3	0.204	A	CS-20	0.359	BC
	32SK-3	0.329	AB	CS-20	0.151	A	32SK-3	0.337	BC
	Water	0.238	B	85SK-1	0.133	A	Water	0.302	C

[a]For treatment details, see footnote of Table 1.

recovered from the roots of plants treated with either the biocontrol strain or one pathogenic strain than from those treated with water alone. Caffeic acid recovered from roots was not different among treatments for either the 24 or 72 h exposures (Table 2). There were no differences among treatments for vanillic acid recovered from roots after 24 h exposure to fungi. After 72 h exposure, more vanillic acid was recovered from plants treated with one pathogenic strain (34SK-3) or one biocontrol strain (85SK-1) than from those treated with water.

At 24 h in roots, levels of UN-324 were lower in biocontrol treatments than the control or pathogen treatments. At 24 h in leaves, significantly ($P \leq 0.05$) more UN-324 was detected in the control treatment than any other treatment (data not shown).

Dosage response – drench

For phenolics recovered from both leaves and roots, there were significant interactions between the biocontrol/no biocontrol treatment and the inoculum level of the pathogen for ferulic and caffeic acids ($P \leq 0.05$). Figs 1–4 illustrate the complex, nonlinear relationships observed. For example, in roots from plants treated with the biocontrol agent and then exposed to different concentrations of the pathogen, both ferulic and caffeic acid were significantly increased relative to the control treatment when the pathogen level was 10 or 10^3 spores/ml, but not when the pathogen was absent or when the pathogen was present at 10^5 spores/ml (Figs 1 and 2). Roots from plants treated with the biocontrol agent and then exposed to different concentrations of the pathogen for only 24 h had increased ferulic acid relative to the control at 10 and 10^5 pathogen spores/ml, but decreased ferulic acid relative to the control at 10^3 spores/ml (Fig. 1). The significantly greater amount of ferulic acid recovered from the roots of plants treated with the biocontrol agent and 24 h pathogen is in contrast to a similar treatment in the experiment in which the biocontrol agent was applied as a root dip rather than a drench. For caffeic acid in roots, the biocontrol treatment was not different at any pathogen level when plants were exposed to the pathogen for 24 h (Fig. 3).

Similar to the root dip experiment, the amount of ferulic acid recovered from leaves was greater than

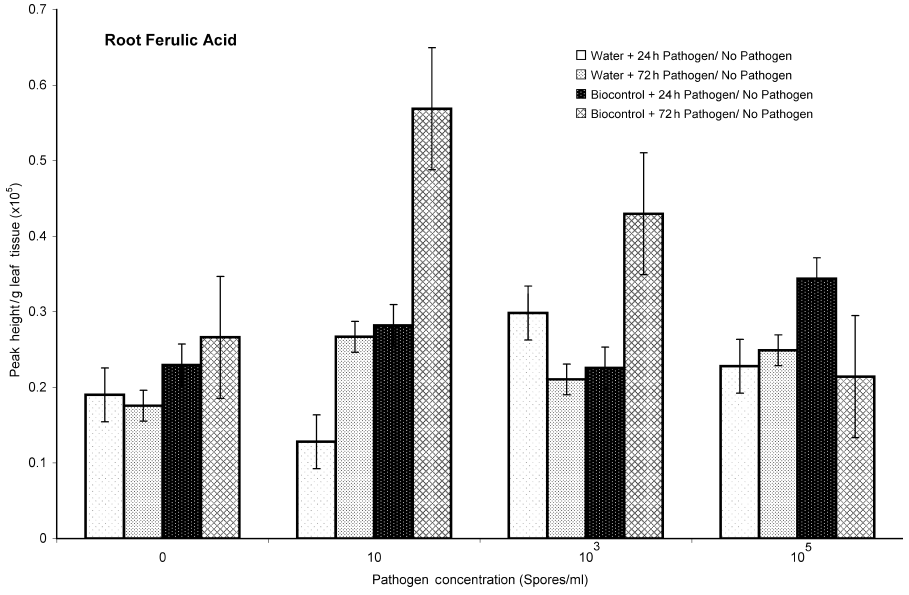

Fig. 1 Ferulic acid recovered from tomato roots. Tomato seedlings grown in sterile sand were drenched with spores of the biocontrol fungus *Fusarium oxysporum* strain CS-20. After 24 h, the seedlings were removed from sand and placed in beakers of either water or a spore suspension of pathogenic *F. oxysporum* f. sp. *lycopersici*. After 24 or 72 h, phenolic compounds were extracted from roots and leaves. These were identified and quantified (relative units, peak height) by HPLC. Bars represent standard error of the mean

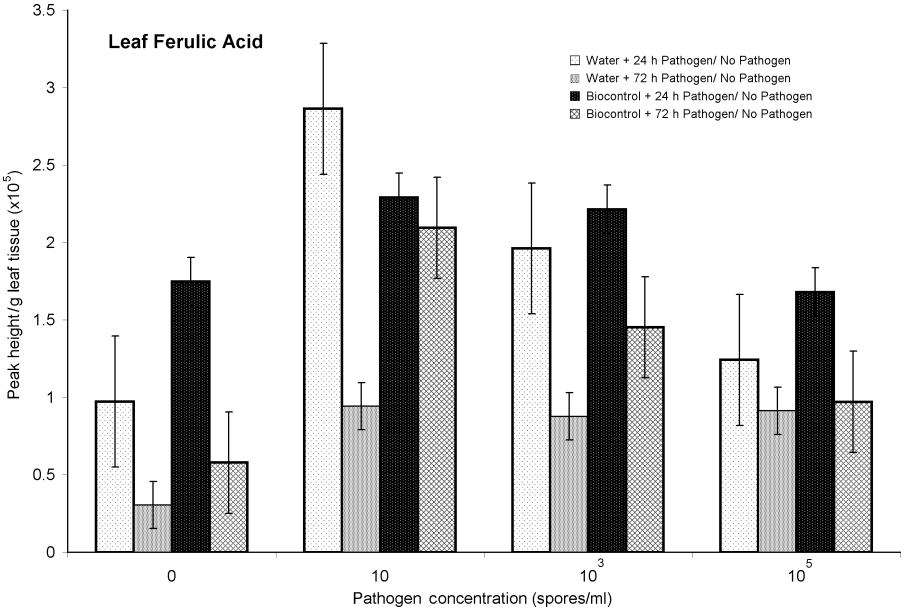

Fig. 2 Ferulic acid recovered from tomato leaves (for details of the treatments, see Fig. 1)

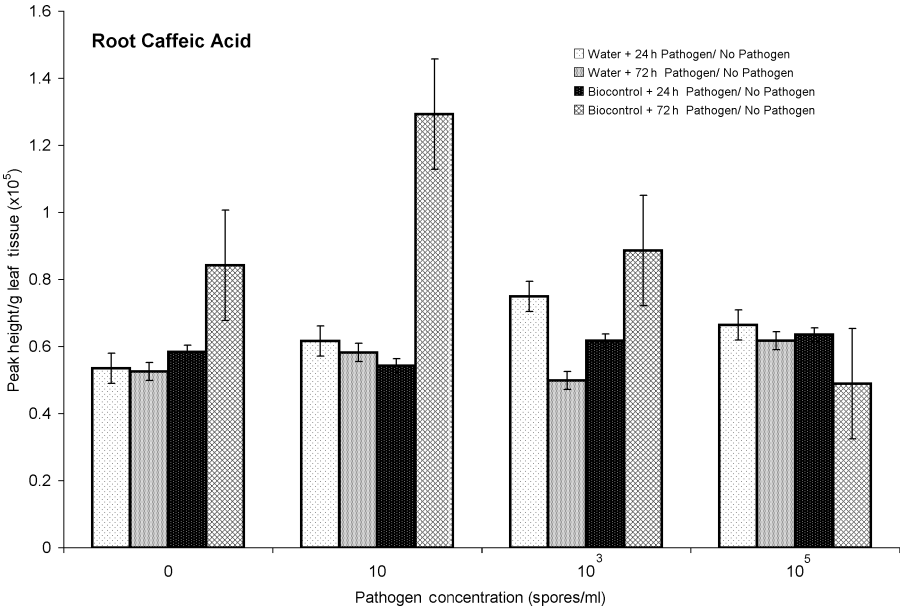

Fig. 3 Caffeic acid recovered from tomato roots (for details of the biocontrol treatment, see Fig. 1)

from plants drenched with strain CS-20 and then exposed to the pathogen for 24 h. These treatments were not different from each other when plants were exposed to the pathogen for 72 h (Fig. 2).

Discussion

As only trace amounts of phenolic compounds were recovered directly from the four *F. oxysporum* strains when they were tested *in vitro*, phenolic compounds recovered from plant assays are presumed to originate from plant tissue. Our research demonstrated qualitative and quantitative changes in phenolics recovered from tomato seedlings following exposure to biocontrol and pathogenic strains of *F. oxysporum*. Detection of changes in phenolic compounds in leaves after exposing roots to the fungi indicates that the response is truly systemic and that the physiological state of the plant has been altered. This is in agreement with reports of increases and decreases in tomato phenolics in response to other biotic and abiotic stressors (Tamietti et al., 1993; Díaz et al., 2005; Cavalcanti et al., 2006). As Duijff et al. (1998) have pointed out, most research in this area has been carried out using young plants, and the results should be confirmed for older plants. As Beckman (2000) has pointed out, the importance of phenolic compounds in reducing wilt diseases may be less in their direct toxicity to the pathogen and more in host defence pathways and in signalling for host defences. Phenolic compounds are building blocks for many secondary metabolites, including those involved in host defence responses.

While most investigators examined total phenolics at a single time point, de Ascensao and Dubery (2003) studied the changes over time in specific phenolic

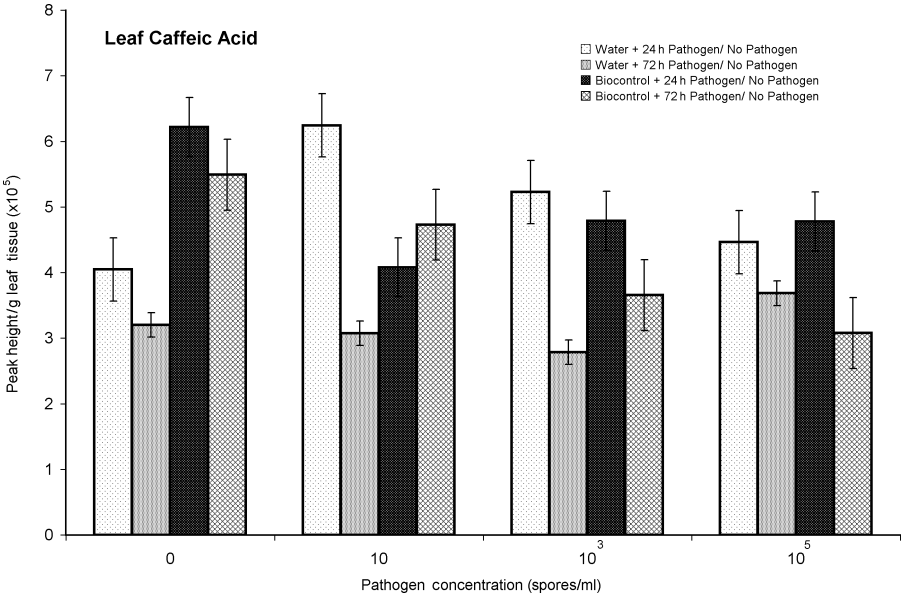

Fig. 4 Caffeic acid recovered from tomato leaves (for details of the biocontrol treatment, see Fig. 1)

compounds in banana in response to the pathogen *F. oxysporum* f. sp. *cubense*. They reported that induced phenolics included P-coumaric, ferulic, sinapic and vanillic acid. They further indicated that that phenolic synthesis was rapid and reached maximum values at 16 h after inoculation. Our results suggest that the time after exposure to the inducer until the maximum amounts of phenolics present may be related to dose, a function of both the quantity of the inducer applied and the duration of the application. As the response appears to change rapidly over time, the same inducer may appear to increase or decrease specific phenolic acids depending on the dosage and the time after dosing that phenolic compounds are measured. The timing of the response can be critical to the success or failure of host defence.

As our experiments were repeated with similar results, apparent discrepancies between measurements at the 10^5 pathogen level in the drenching experiment compared with the root dip experiment for ferulic acid recovered from roots and caffeic acid from leaves should be considered real effects. These differences may be due to the dose effect, as well as the root dip vs. drench method of applying strain CS-20.

Reddy et al. (1999) reported protection of wheat from *F. graminearum* and synthesis of phenolics, especially ferulic acid, in primary leaves following the treatment of wheat seed with chitosan. Similarly, the biocontrol fungus *Pythium oligandrum* induces phenolic compounds, particularly ferulic acid, protecting wheat from *F. graminearum* (Takenaka et al., 2003). Genetic resistance to *F. graminearum* in wheat has also been linked to ferulic acid (Bily et al., 2003). Benhamou et al. (1994) reported induced resistance to Fusarium root and crown rot in tomato following a chitosan seed treatment. He and Wolyn (2005) reported the induction of salicylic acid and lignification in asparagus following inoculation with both pathogenic and non-pathogenic *F. oxysporum*.

Our result indicated that the timing and magnitude of the host response may differ depending on the strain of the fungus inducing the response. Similarly, Benhamou and Garand (2001) found that defence responses triggered in pea plants were different when plants were treated with the pathogen *F. oxysporum* f. sp. *pisi* compared with the biocontrol fungus *F. oxysporum* strain Fo47. The pattern of electron density of particles surrounding hyphae in the cytological analysis of these pea plants indicates the induction of phenolic compounds by strain Fo47.

As broad-spectrum fumigants become less available, environmentally benign management tools are needed for use against soilborne plant pathogens. Knowledge of the mechanisms of biocontrol and of induced resistance may lead to reliable alternatives for disease management. Bound phenolic compounds are quickly activated in response to various stresses. Additional research is needed to further understand the temporal and quantitative similarities or differences in host responses to these stresses so that the response can be elicited when it is needed for host defence.

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Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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